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14. ABSTRACT

Parkinson's disease (PD) is characterized by progressive loss of dopaminergic neurons in the nigrostriatal pathway resulting in significant motor dysfunction. The pathology of PD is mimicked by exposure to 1-methyl-4-phenyl-1,2,3,tetrahydropyridine (MPTP) or the pesticide rotenone. These neurotoxins inhibit complex I of the mitochondrial respiratory chain resulting in the production of reactive oxygen species (ROS) and increased cytosolic calcium. We hypothesize that ROS promotes opening of the mitochondrial permeability transition pore which triggers the death pathway. In parallel, increases in cytosolic calcium leads to oxidative stress and activation of c-Jun-NH2-terminal kinase (JNK). JNK/c- Jun signaling augments activation of the mitochondrial apoptotic cascade by suppressing Bcl-2 pro-survival signals via phosphorylation of Bcl-2 or transcription of the BH3-only, Bcl-2 antagonist Bim. The interactions between the oxidative stress pathway, the JNK/c-Jun signaling cascade, and the mitochondrial apoptotic machinery ultimately determine the fate of dopamine neurons. We will utilize primary ventral mesencephalic cultures obtained from E15 embryonic rats to investigate our hypothesis. The data obtained should lead to the identification of promising therapeutic strategies to slow or halt the dopaminergic neurodegeneration that occurs during progression of PD.

15. SUBJECT TERMS
neurodegeneration, apoptosis, dopamine neurons, MPTP, rotenone, intrinsic death pathway

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Table of Contents

Annual Progress Report- #W81XWH-04-1-0001, "Signaling Pathways that Mediate Neurotoxin-induced Death of Dopamine Neurons". November 1, 2006-October 31, 2007

Introduction

The overall goal of our studies is to better understand the molecular mechanisms that control neuronal death so that we can identify new therapeutic targets for neurodegenerative diseases particularly Parkinson's disease. Our approach is to study dopamine neurons in culture where we try to create *in vitro* conditions that are relevant to neurodegenerative disease. That is to say, we create conditions that cause the death of dopamine neurons in order to understand what cellular proteins mediate death and how neurotrophic factors (GDNF) prevent death.

Our overall working hypothesis is that the Parkinsonian neurotoxins, MPP+ and rotenone, share a common mechanism of action to induce death in dopaminergic neurons. By inhibiting complex I of the mitochondrial respiratory chain, these toxins result in the production of reactive oxygen species (ROS) that leads to opening of the mitochondrial permeability transition pore (mitoPTP) and activation of a JNK/c-Jun signaling cascade. Opening of the mitoPTP induces Bax translocation to mitochondria and Bax-dependent cytochrome C release that initiates the intrinsic apoptotic cascade. Activation of the intrinsic death pathway is augmented by JNK/c-Jun-dependent inhibition of Bcl-2 pro-survival signals. GDNF prevents cell death by regulating the activity the death machinery.

To study primary differentiated dopamine neurons *in vitro*, we culture cells obtained from embryonic rat or mouse ventral mesencephalon (the location of most dopamine neurons in the CNS). Although we are successful in obtaining healthy, differentiated dopamine neurons from ventral mesencephalon, the percentage of dopamine neurons in the cultures is very low (generally less than 3% of total cells). A second problem with the mesencephalic cultures centers on methods that are currently available to identify dopamine neurons in culture. To date, most investigators stain the cultures for tyrosine hydroxylase (TH, the rate-limiting enzyme in the dopamine biosynthetic pathway) to identify dopamine neurons. The staining procedure, however, kills the neurons and limits the types of experiments that can be done. To circumvent this issue, we have used a GFP-TH reporter construct to identify living dopamine neurons in culture. This allows us to study dopamine neurons (GFP-positive cells) using live-cell imaging techniques. Most of our data obtained in the first two years of study was obtained by delivering the GFP-reporter construct using the Helios gene-delivery system. This method works for dopamine neurons, however, the yield of transfected cells is low (less than 5%). The low transfection efficiency combined with the low numbers of dopamine neurons in the cultures has made it difficult to obtain enough single-cell measurements for quantitative analysis. For these reasons we have spent a lot of effort over the past year investigating new ways of transfecting dopamine neurons. Results from these efforts and progress in our original aims are described below.

Note: Due to personnel turnover, a new technician (with expertise in primary culture) and a new postdoctoral fellow (an experienced molecular biologist) were brought on board this funding funding period.

Task 1. To determine the effects of the neurotoxins, MPP+ and rotenone, on components of the mitochondrial apoptotic cascade in primary neurons.

Most of the experiments under this task have been described in previous reports.

Task 2. To investigate the role of reactive oxygen species and nitrogen species in neurotoxin-induced activation of the mitochondrial apoptotic pathway.

Experiments in the last year have indicated that rotenone-induced death of dopamine neurons is completely reversed by the addition of n-acetylcysteine (NAC) to the cultures at the time of rotenone addition (see below). This supports out hypothesis that ROS is an activator of the intrinsic apoptotic cascade. The effect of NAC is seen at high doses of rotenone under conditions where GDNF is unable to protect against death (explained in more detail under Task 4). Once we have a more efficient way of labeling live dopamine neurons (see progress described below), the generation of ROS induced by rotenone (and MPP+) will be measured in GFP-positive dopamine neurons using the redox-sensitive dye CM-H2-DCFA (Molecular Probes) in the absence and presence of various inhibitors to determine the relationship between ROS and mitochondrial mPTP opening.

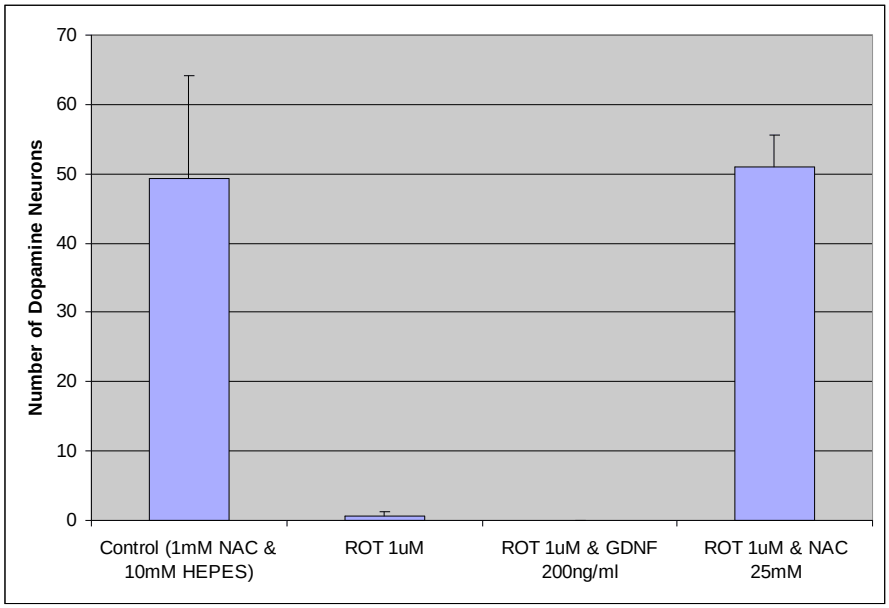


Figure 1. Primary cultures derived from E15 rat ventral mesencephalon were prepared in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution by mechanically dispersing tissue pieces. Subsequently, cells were centrifuged and resuspended in F12 medium containing 5% heat-inactivated human placental serum, 2 mM l-glutamine, 100 ug/ml streptomycin, 100 U/ml penicillin. Cells were seeded on polyethylenimine-coated 24-well cover slips in 0.1 ml of medium at 60000 viable cells/cm^2. Culture medium was changed every two days. On day 4, cultures were treated with or without rotenone (48hr) in the absence or presence of NAC. After treatment, cells were fixed with 4% paraformaldehyde and then permeabilized and blocked in PBS containing 0.2% Triton X-100 and 5% bovine serum albumin. Cells were then incubated overnight at 4 °C with monoclonal mouse anti-tyrosine hydroxylase (TH, 1:500). The primary antibody was aspirated, and cells were washed five times with PBS. Cells were then incubated with the appropriate Cy3-conjugated antibody (diluted 1:500) and DAPI for 2 h at room temperature. The cells were then washed 5 times with PBS, and cover slips were adhered to glass slides with mounting medium (0.1% *p*-phenylenediamine in 75% glycerol in PBS). Fluorescence measurements were obtained on Zeiss Axioplan 2 microscope equipped (63x fluorite objective) with a Cooke Sensicam deep-cooled CCD camera.

Task 3. To determine if neurotoxin-induced oxidative stress signals through stress-activated protein kinase pathway to activate mitochondrial apoptotic death in primary neurons.

The ability of rotenone and MPP+ to activate components of the JNK signaling cascade using immunocytochemical techniques (co-staining for TH and active phospho-kinases) are complicated by the fact that we now know that both rotenone and MPP++ kill dopamine neurons by more than one death mechanism. We will need to do detailed dose-response curves to both neurotoxins to determine if the JNK signaling cascade is associated with apoptotic death vs. death associated with ATP depletion. Our improved culture methods using anti-oxidants during tissue dissection and dissociation and plating the cells on laminin have quadrupled the number of TH+ neurons per well. This will cut down on the number of slides that need to be counted in order to obtain statistically significant data. We will perform these experiments in the no-cost extension period of this grant.

Task 4. To elucidate the neuroprotective effects of GDNF in primary dopamine neurons exposed to neurotoxins.

We have determined that GDNF exerts two actions in ventral mesencephalic cultures. The first effect is a trophic or survival effect on dopamine neurons. When GDNF is added to the cultures at the time of plating (even in the presence of serum), approximately 50 % more dopamine neurons survive in culture (see below). This effect is selective to dopamine neurons. The second effect of GDNF is to protect dopamine neurons from MPP+ (see below) and rotenone toxicity (not shown).

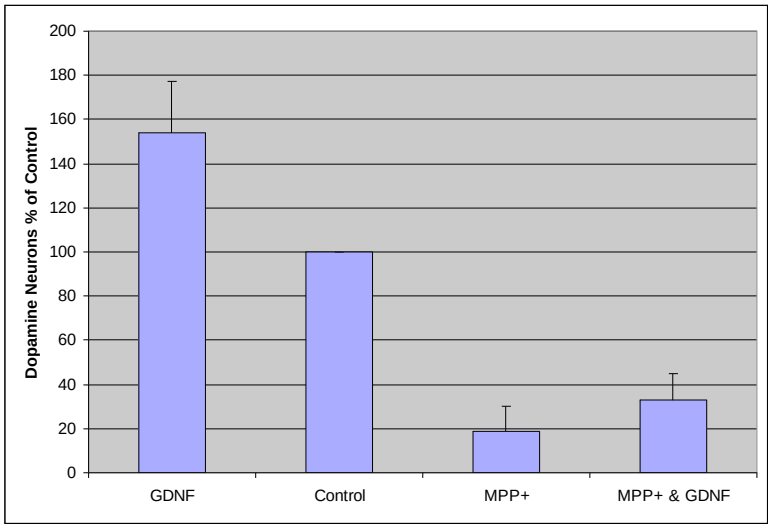


Figure 2. Primary cultures derived as described in Figure 1 except GDNF (200ng/ml) was added to the culture medium in on experimental group (column 1). On day 4, cultures not subjected to GDNF were treated with or without MPP+ (48hr) in the absence or presence of GDNF. After treatment, cells were fixed and stained with monoclonal mouse anti-tyrosine hydroxylase as described in Figure 1. Fluorescence measurements were obtained on Zeiss Axioplan 2 microscope equipped (63x fluorite objective) with a Cooke Sensicam deep-cooled CCD camera.

The neuroprotective effects of GDNF on MPP+ exposure have been variable and appear to depend on the dose of neurotoxin used. This, is turn, is related to the type of cell death that occurs after rotenone and MPP+ exposure. At low doses of both toxins, death occurs largely by an apoptotic mechanism dependent on the presence of ATP. Once ATP is depleted as a result of inhibition of complex I, the neurons die by a non-apoptotic mechanism that is insensitive to GDNF.

Even at low doses of MPP+, the protection by GDNF (200 ng/ml) is never complete. During the course of these experiments, the PI read a report that the neurotrophic actions of GDNF require TGF-beta. Experiments were carried out to determine if pretreatment or addition of TGF-beta with GDNF would enhance the neuroprotective effects of GDNF following MPP+ or rotenone exposure. The results were conclusively negative. TGF-beta had no effect on the ability of GDNF to protect against MPP+ or rotenone in ventral mesencephalic cultures.

Efforts to increase the delivery of the TH reporter construct and other genes into primary dopamine neurons.

I. Amaxa Nucleofector Technology using rat ventral mesencephalic neurons.

Many laboratories, including the PI's, have had success using Nucleofector technology for gene-transfer in primary cells and hard-to-transfect cell lines. The technology involves modified electroporation parameters and specific solutions that permit gene transfer into the nucleus. We have been successful in optimizing conditions that result in 60-70% transfection efficiency of primary cerebellar granule neurons. Conditions that proved successful for granule neurons were found to be toxic for dopamine neurons in ventral mesencephalic cultures. We tried different settings (Amaxa programs G13 and O-03) and different cell densities and had no success with dopamine neurons. Last year, Amaxa representatives introduced us to a new 96-well shuttle system that has many more electrical programs available to the user and a modified cuvette that apparently results in less cell toxicity. With the assistance of Amaxa technicians, we spent 2 months investigating parameters (DNA quality, cell density, different proprietary solutions, and multiple electrical programs) that would result in efficient transfection of primary dopamine neurons. These were labor-intensive experiments since they required 2-4 million cells per reaction. We found that all of the optimized programs used for other cell types, including other types of primary neurons, lead to the death of dopamine neurons, assessed by TH staining. A few conditions that resulted in transfection of non-dopamine cells and low toxicity to dopamine neurons, resulted in no transfection of dopamine neurons. At 2-4 million cells per reaction, we decided to abort these experiments, although the Amaxa technicians were confident that the nucleofector technology would eventually be successful for dopamine neurons.

II. Exploration and development of viral vectors for gene delivery to dopamine neurons.

Upon hiring a new molecular biologist on the project and the recent establishment of a viral core in the Neuroscience program, we focused on the use of viral vectors to establish a more efficient

and reliable method for the identification of live dopamine neurons in primary culture. We developed a new strategy based on lentiviral transformation of rat mesencephalic cells using vectors containing a TH reporter driving the expression of GFP.

A lentiviral expression system was chosen over adenoviral and adeno-associated virus (AAV) expression vectors to deliver the GFP-TH reporter construct into primary dopamine neurons because lentiviral transduction resulted in the lowest amount of cellular toxicity and the highest amount of expression of GFP in preliminary studies (see Figure below). Adenoviral GFP expression vectors were very toxic to dopamine neurons, although we use them for other primary neurons in the lab. AAV transduction was not toxic to dopamine neurons, but transduction efficiency was less than with lentivirus and AAV is more limited by insert capacity (less than 4.5 kb). The major challenging factor in making a lentiviral GFP-TH expression vector relates to the fact that lentiviral vectors can only incorporate DNA fragments less than 8 kb and the established 9 kb rat promoter coupled to GFP (Patankar *et al.,* 1997) is consequently too large.

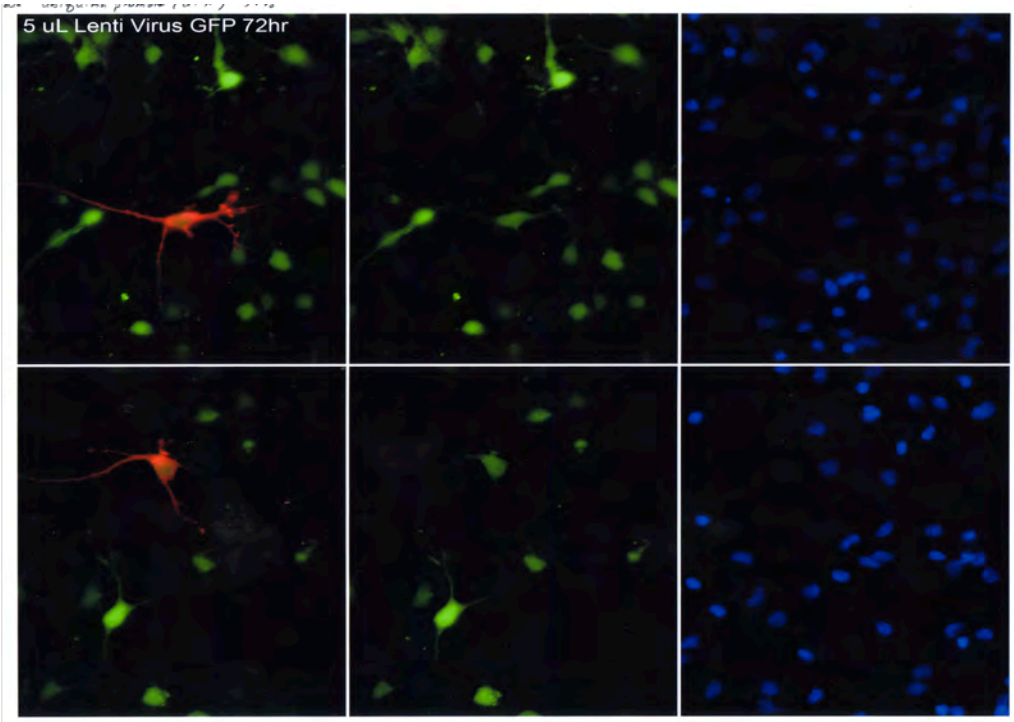
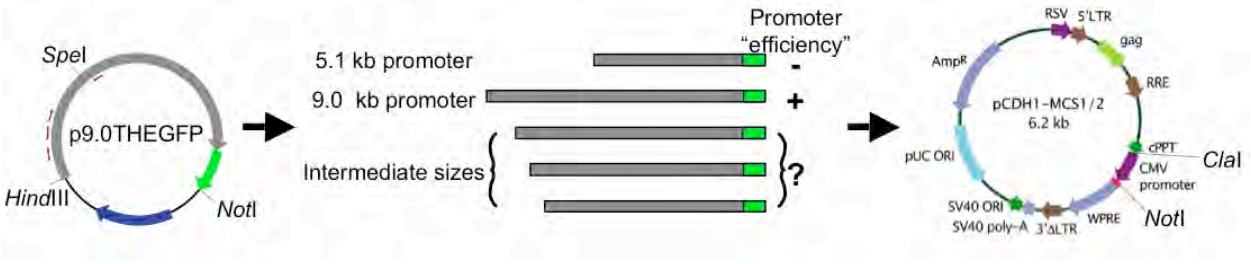


Figure 3. Lentiviral transduction of dopamine neurons. E15 rat ventral mesencephalic cultures (3 days in culture) were infected with a lentivirus -GFP at a MOI of approximately 50. Seventy two hours later, the cultures were fixed and immunostained with a primary antibody against TH (rabbit polyclonal from Pel-Freeze) followed by a secondary goat anti-rabbit antibody conjugated to Alexa Fluor555 and DAPI. Left panels display TH staining and GFP expression, middle panels display GFP alone, and right panels display DAPI alone. Dopamine neurons (shown in red) were GFP positive.

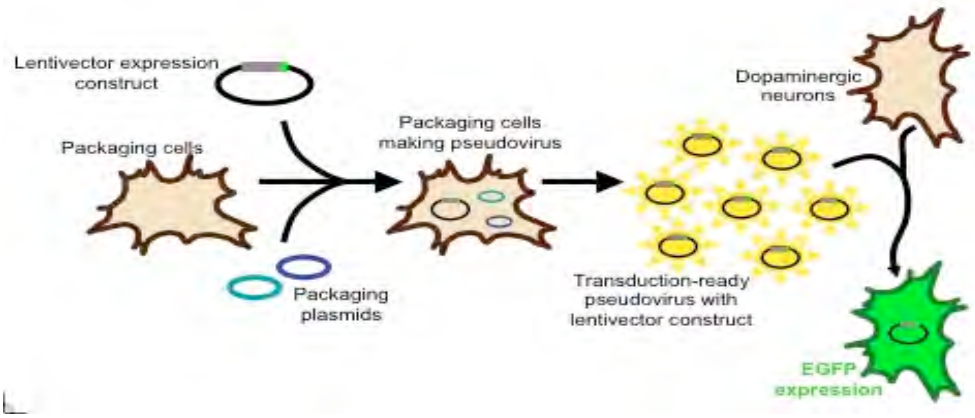
Preliminary experiments showed that a 9kb (8660 bp) but not a 5kb (5280 bp) TH promoter generates GFP expression in primary dopamine neurons. We hypothesized that generation of fragments of intermediate length will successfully work as TH reporter constructs in lentiviral vectors. Our hypothesis was supported by the fact that a putative binding site for a transcription factor of the Pitx family, expressed in the midbrain at ED 10.5, is found at the position – 6840

(Sclafani *et al*., 2006). To optimize our chances of obtaining a useful lentiviral vector that will deliver an efficient TH-GFP reporter construct, we designed a pool of sequences of intermediate sizes ranging from 6.9 to 8 kb. The cloning strategy that was used to obtain these mutant clones is shown on the figure below.



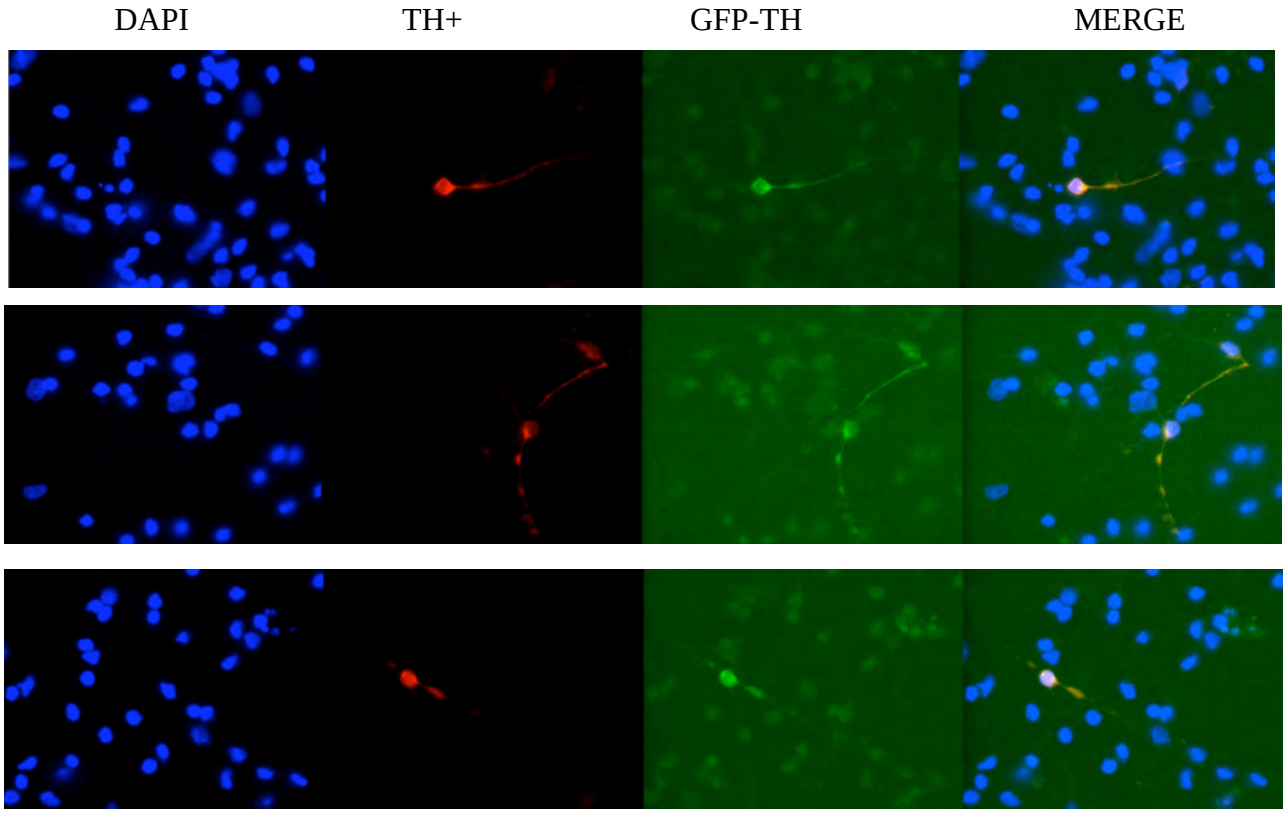
The starting vector p9.0THEGFP, containing the GFP coding sequence downstream of the 9.0 kb rat TH promoter served as the template for several PCR amplifications, in which the primers located in the distal part of the promoter contained at their 5' extremity an additional *Hind*III restriction site. This allowed the use of the *Hind*III and *Spe*I unique restriction sites to re-insert the amplified fragments in the p9.0THEGFP plasmid cut beforehand with the same enzymes and gel purified. This procedure resulted in the construction of 3 new pEGFP plasmids carrying TH promoters of 6.8, 7.3 and 7.8 kb. After complete sequencing of the new constructs, the following stage involved cutting the promoter-GFP fusions obtained using the *Hind*III and *Not*I restriction sites, in order to isolate the genetic material to be transferred to the lentiviral expression plasmid. This pCDH1 plasmid was cut using the *Not*I and *Cla*I restriction sites, in order to remove the constitutive CMV promoter. Since the *Hind*III and *Cla*I restriction sites are not compatible, we needed to blunt the single strand extremities of the purified fragments, using the T4 DNA polymerase. Finally, after a dephosphorylation step carried out to avoid the recircularization of the pCDH1 vector without insert, the promoter-GFP fusions and the lentiviral expression plasmid deprived of the CMV promoter were subjected to ligation. Unfortunately, the blunt ligations never worked. We re-designed the cloning strategy by inserting an adapter in the lentiviral expression plasmid, which allowed the use of cohesive restriction sites. This approach was successful and yielded three new lentiviral plasmids early this fall.

As shown in the next schematic, the next step of the project consisted of packaging the newly obtained expression constructs into pseudo viral particles.



For this purpose, we used the pPACKH1 Lentivector Packaging Kit from System Biosciences. Briefly, 293TN human kidney producer cell lines were co-transformed with our different expression constructs and the pPACKH1-GAG, pPACKH1-REV, and pVSV-G packaging plasmids. Following the co-transformation, the pseudo-viral particles obtained were collected, purified, and titrated.

We are now in the process of screening the viruses to determine which promoter length results in the highest amount of dopamine neurons expressing GFP. Preliminary results using the intermediate sized TH promoter virus are shown below.



We can detect transduction of dopamine neurons, assessed by staining with TH antibodies. However, we have encountered a problem of high auto fluorescence (background green staining seen in 3rd panel). Experiments are being carried out to increase signal-to-noise ratio by decreasing auto fluorescence, concentrating the three different viruses to increase transduction efficiency, and comparing transduction seen with all three new GFP-TH-lentiviral vectors.

Key Research Accomplishments

Since the start of this project, we have been frustrated by the low number of labeled dopamine neurons that result from Helios-gene gun delivery. This technique can be used to obtain qualitative single-cell measurements, but we have found does not label enough cells for quantitative analysis. Therefore, we made the decision to focus part of our effort on making a useful reagent for our own studies and making this reagent available for other investigators in the field. The key research accomplishment this year has been our progess is making a lenti-viral GFP-TH reporter construct.

Reportable Outcomes

Publications

Brewster JL, Linseman DA, Bouchard RJ, Loucks FA, Esche E, Precht T, and **Heidenreich KA**. Endoplasmic reticulum stress and trophic factor withdrawal activate distinct signaling cascades that converge at GSK-3β to trigger mitochondrial apoptosis in neurons. Mol. Cell. Neurosci. 32:242-253, 2006**.**

Precht, T and **KA Heidenreich**. The role of mitochondrial fission in regulating intrinsic apoptosis of neurons. Society for Neuroscience, Atlanta, October, 2006.

Precht, TA, J Schaak, and **KA Heidenreich**. Mitochondrial fission plays a role in neuronal apoptosis during trophic factor withdrawal. Rocky Mountain Neuroscience Group Meeting, Aurora, CO, May, 2007. Poster award.

Zaegel, V and **KA Heidenreich**. A Novel Strategy for Identifying Live Dopamine Neurons. Pharmacology Retreat, Copper Mountain, CO, October, 2007

Grants Funded

VA Merit Award "Autophagic Cell Death and Neurodegeneration""
K.A. Heidenreich – Principal Investigator
Total Award: $550,000 (10/01/06-9/31/09)

Invited talks

2007	KA Heidenreich, Invited speaker, Gordon Research Conference, Insulin-like Growth Factors in Physiology and Disease, Venture, CA (canceled due to illness)
2007	KA Heidenreich, Department of Pharmacology, University of Texas Health Science Center, San Antonio, TX, “Different Ways that Neurons Die”.

Conclusions

Our research this year resulted in a better understanding of the complexity of how rotenone and MPP+ kill dopamine neurons. The single-cell experiments designed to provide details of how ROS leads to activation of the intrinsic apoptotic program in dopamine neurons have been delayed while we attempted to improve gene delivery methods for dopamine neurons. So far, the results with the lentiviral TH-GFP reporter look promising and may yield a useful reagent for work in this field.

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Patankar S, Lazaroff M, Yoon SO, Chikaraishi, DM: A novel basal promoter element is required for expression of the rat tyrosine hydroxylase gene. J. Neuroscience 17:4076-4086, 1997.

Sclafani AM, Skidmore JM, Ramaprakash H, Trumpp A, Gage PJ, Martin DM: Nestin-Cre mediated deletion of Pitx2 in the mouse. **Genesis** 44(7):336-44, 2006.